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Genetic Analysis of Erythrocyte Binding Antigen 175 (EBA-175), Apical Membrane Antigen (AMA-1) and Merozoite Surface Protein 3 (MSP-3) Allelic Types in Plasmodium Falciparum Isolates From Rural Area in Senegal

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Abstract

Background: Several of the intended *P. falciparum* vaccine candidate antigens are highly polymorphic and could render a vaccine ineffective if their antigenic sites were not represented in the vaccine. This study aimed to characterize genetic diversity of vaccine candidate antigens merozoite surface protein-3 (MSP-3), apical membrane antigen-1 (AMA-1) and erythrocyte binding antigen (EBA-175) in *P. falciparum* isolates from Senegal.

Methods: DNA analysis was completed on 170 isolates of *P. falciparum* collected from Keur Soce in Senegal between 2006 and 2008. Genetic diversity was determined in the three *P. falciparum* genes by, PCR followed by restriction fragment length polymorphism (RFLP).

Results: From 170 samples collected, successful, PCR products were obtained from 135 (79%), 140 (82%) and 128 (75%) for AMA-1, MSP-3 and EBA-175, respectively. The results showed that the EBA-175 gene presented 4 different alleles [EBA-175F_loop (62.3%), EBA-175C_loop (46.1%), EBA-175~400bp (17.6%), EBA-175~360bp (8.4%)]. Regarding the MSP-3 patterns, the analysis revealed the presence of three alleles MSP-3_K1 (49.2%), MSP-3_3D7 (54.2%) and MSP-3~350bp (15%). For AMA-1, the results showed three different alleles AMA-1_K1 (39%), AMA-1_HB3 (33%), AMA-1_3D7 (32%).

Conclusion: Characterization of the genetic diversity in *Plasmodium* isolates from Keur Soce in Senegal in the three genes investigated showed a high degree of polymorphism. These findings are helpful in the formulation of a vaccine considering restricted repertoire populations.

Keywords: Malaria; *Plasmodium falciparum*; Genetic diversity; MSP3; EBA-175; AMA-1; Vaccine

Background

Malaria remains a major public health problem in tropical regions. In most African countries such as Senegal, the vast majority of cases and deaths occur in young children [1]. Currently key interventions recommended by WHO for the control of malaria are the use of Insecticidal Treated Nets (ITNs) or Indoor Residual Spraying (IRS) for vector control, and prompt access to diagnosis and treatment of clinical malaria by using Rapide Diagnostic Test (RDT) and Arthemisinin Combination Therapy (ACT) respectively [2]. For malaria prevention, an additional strategy such as Intermittent Preventive Treatment (IPT) is recommended specifically in risk group (pregnancy women and infancy) in area of high malaria transmission [2]. In areas of markedly seasonal malaria transmission, such as the Sahel and sub-Sahel regions of Africa, the main burden of malaria is in older children rather than infants, and the risk of clinical malaria is restricted largely to a few months each year [3,4]. In such areas, administration of IPT to children (IPTc named seasonal malaria chemoprevention (SMC) 3 months to 5 years of age monthly during the seasonal peak in malaria is recently recommended by WHO [5]. All these strategies have met with some success due to the emergence and spread of drug resistant parasites. However the challenge of developing malaria elimination which has been proposed recently due to the declining of malaria in many African countries such as Senegal, will probably be accomplished by the development of an effective vaccine in addition of vector control strategies [6]. The complexity of the malaria parasite life-cycle stages combined with an exuberant genetic diversity constitute major problem to the development of a successful malaria vaccine [7,8]. The antigen polymorphism greatly enhances the parasites ability to evade immune recognition, making it difficult to bring forth adequate responses against variants of the circulating parasite population [9]. Fluctuations in genetic diversity across transmission seasons can further complicate controlling the disease [10-12]. It is therefore important to understand the genetic diversity in polymorphic antigens in endemic populations before the introduction of any malaria vaccine, including the prevalence of different genetic variants and their natural dynamics. Several *P. falciparum* stage-specific antigens such as the erythrocyte binding antigen 175 (EBA-175), the apical membrane antigen (AMA-1) and the merozoite surface protein 3 (MSP-3) have been suggested as vaccine candidates through molecular epidemiological studies [13]. However, the comparative distribution of the malaria parasite genotypes circulating across a wide geographical endemic area provides important genetic information which could be helpful for malaria vaccines design. The AMA-1 is expressed in the late schizont

stage of the parasite and is required for both merozoite invasion of erythrocytes and sporozoite invasion of hepatocytes. Recombinant AMA-1 induces protective immune responses in mouse and monkey models of malaria [7,8] and both monoclonal and polyclonal antibodies to AMA-1 inhibit merozoite invasion of erythrocytes [9]. Antibodies raised against AMA-1 have been shown to block parasite invasion of human red blood cells. The main concerns related to AMA-1 as a vaccine candidate is the fact that Pfama-1 is highly polymorphic with most of polymorphisms occurring in domain I [10-13] making a broadly effective vaccine difficult to create. *Plasmodium falciparum* uses also a 175-kDa sialic acid binding protein ligand, known as erythrocyte binding antigen EBA-175, for erythrocyte invasion [14, 15]. EBA-175 is a potential vaccine candidate as it induces antibodies which inhibit malaria merozoite invasion. The gene encoding EBA-175 is well established to be dimorphic the FCR-3 (referred to as the F loop and cAMP (referred to as the C loop) strains of *P. falciparum* [16]. The role that dimorphism plays in host-parasite interactions (for example, potential difference in efficiency of red blood cell invasion between genotypes) remains unclear.

Another antigen that is considered as vaccine candidate for *P. falciparum* is the MSP-3 protein which is encoded by a single locus on parasite chromosome 10. MSP-3 is an important target for protective immunity as antibodies against it could also prevent erythrocyte invasion by merozoites [17]. Previous analysis of the MSP-3 gene from *P. falciparum* isolates shows that polymorphism in the gene is predominantly due to sequence in the N-terminal extremity within and flanking the heptad-repeat identified as a site of antigenic diversity among MSP-3 polypeptides [18]. The C-terminal domain (corresponding to amino acids 196–379 in the K1 allele sequence) is highly conserved among various parasites isolates [19]. There are several sequence differences among MSP-3 alleles, but the sequence polymorphism defined 2 major allele types (MSP-3_K1 and MSP-3_3D7), with only very limited recombination [20].

The purpose of this study is to determine in the context of malaria vaccine development, the diversity of three *P. falciparum* vaccine antigens candidate (AMA-1; EBA-175 and MSP-3) in Senegal. To accomplish this, *Plamodium falciparum* field isolates collected from endemic area in Senegal during year 2006 to 2008 were used to assess the frequency of the major alleles of these three candidates vaccines antigens by PCR-RFLPs. Knowledge of the distribution of polymorphic malaria antigens may contribute to rational vaccine development.

Material and Methods

The study took place between 2006 and 2008 in the rural community of Keur Soce located some 200 km south-east of Dakar and 17 km south of Kaolack city in the center of Senegal. Malaria transmission in this locality is seasonal with entomological inoculation rates varying between 9 and 12 infected bites per person per night during the rainy season from September to November (unpublished data from Kanate L et al.). The population is about 20,415 inhabitants and 20% of the population is children under five years old. In this locality, malaria morbidity was 35% at high transmission season from September to November during our study period.

Samples collection

Samples were collected at Keur soce rural area during a clinical trial study comparing once daily Artesunate plus Amodiaquine (AS/AQ) or twice-daily Artemether plus Lumefantrine (AL) [21] from patients of any age with symptoms of uncomplicated *P. falciparum* malaria. A total number of 170 subjects infected with *P. falciparum* at day zero living in Keur Soce area were included in the study. Thick and thin blood films were done for *P. falciparum* microscopical identification. Whole blood from *P. falciparum* positive subjects were blotted on sterile, Whatman 3 MM filter paper and stored at room temperature protected with silica gel in individual plastic bags for *P. falciparum* DNA extraction.

Microscopic examination

Giemsa stained blood smears were microscopically examined to identify mono-infections with *P. falciparum*. The slides were independently examined by two experienced microscopists. An individual was considered positive if malaria parasites were detected in the blood smear and negative if parasites were not detected after examining 200 oil-immersion fields of the thick smear. The parasite density was determined by multiplying the number of parasites present per 200 white blood cells in a thick smear by 40 to arrive at an approximate parasite count per microliter of blood. This was based on the assumption that the average WBC count was 8,000/l blood.

P. falciparum DNA extraction

Extraction of *P. falciparum* DNA from positive filter paper was carried out by means of the QIAmp DNA blood mini kits (manufacture Qiagen, www.quiagen.com/gDNA). Extraction via the Qiagen kit method followed the manufacturers' protocol [22]. All DNA samples were stored at -20°C for PCR genotyping.

PCR amplification

Nested and semi-nested polymerase chain reaction (PCR) methods were applied to analyze polymorphism of EBA-175 and MSP-3 respectively [23]. The 25 µL of EBA-175 or MSP-3 outer PCR mixture consisted of 4 µl of extracted DNA, in 13 µl of water, 2.5 µl of 1X buffer, 1.0 µl of 2.0 mM $MgCl_2$, 1.5 µl of 200 µM dNTP mix, 2 µl of primers [(159F/745R for MSP-3) or (EBA1 /EBA1 for EBA-175)] and 1 unit of Platinum Taq polymerase. For the nested and semi-nested mixture, 2.5 µl of outer of each reaction and the primers set EBA3/ EBA4 and 188F/745R was used for EBA-175 and MSP-3 respectively. Genomic DNA preparation K1 and 3D7 were included as references for both genes.

The ama-1 haplotypes were analyzed by PCR-RFLP method [24]. The 50 µl of AMA-1 outer PCR mixture consisted of 4 µl of extracted DNA, 34 µl of water, 5 µl of 1X buffer, 2.0 µl of 2.0 mM $MgCl_2$, 2 µl of 200 µM dNTP mix, 2 µl of primers VM785/3 /VM990 and 1 µl of Platinum Taq polymerase. For the nested PCR mixture, 2.5 µl of the outer and the primers VM815/3/VM990 was used.

All the reactions were performed using PCR 96 wells micro-plates (sigma manufacture, www.sigma.com) on a classic Thermocycler 96 wells (PTC-100). Primers sequences of each gene for the outer and nested PCR and cycling conditions were listed in Table 1.

P. falciparum MRA-102, MRA-149, MRA152, MRA153, MRA157, MRA 159, MRA 176 and MRA731 lines provided by MR4 (www.mr4.org) were used as positives controls during the amplification reactions. The nested PCR products were directly separated on a 2.0% ethidium bromide stained agarose gel and visualized on GEL LOGIC 220 UV-translumination cabine. 12 µl of the nested AMA-1 product

was digested with 4 µl of the three different restriction enzymes: MseI, SspI, and BfCUI specific to 3D7, K1 and HB3/7G8 AMA-1 haplotypes groups respectively.

Primer name	Sequence length 5'-----3'	Conditions cycling
EBA-175 1st round 2 hr 10 m	EBA1: CAAGAAGCAGTTCCTGAGGAA EBA2: TCTCAACATTCATATTAACAA TTC	94°C x 1:30m 94°C x 30 s, 54°C x 30 s, 68°C x 2.0 m - 35 cycles 68°C x 5 m 4°C x Hold
EBA-175 2nd round 2 hrs 20 m	EBA3: GAGGAAAACACTGAAATAGCACAC EBA4: CAATTCCTCCAGACTGTTGAACAT	94°C x 1:30 m 94°C x 30 s, 54°C x 30 s, 68°C x 2.0 m - 40 cycles 68°C x 5 m 4°C x Hold
MSP 3 1st round 2 hrs 45 m	159F: ATGTTGCTAGTAAAGAAATTG 745R: CATAACTAGAAGCTTCTTTTGC	94°C x 1:30 m 94°C x 30 s, 54°C x 30 s, 68°C x 2.0 m - 35 cycles 68°C x 5 m 4 °C x Hold
MSP 3 2nd round 3 hrs	188F: ATAATCTTAACTTAAGAAATGC 745R: CATAACTAGAAGCTTCTTTTGC	94°C x 1:30 m, 94°C x 30 s, 54°C x 30 s, 68°C x 2.0 m - 40 cycles 68°C x 5 m 4 °C x Hold
AMA 1 1st round 2 hrs 20 m	VM785/3: CCGGATCCCCTTTGAGTTTACATATATG VM990: AATTCTTTCTAGGGCAAAC	95°C x 2:30 m 94°C x 30 s, 51°C x 30 s, 68°C x 45 s - 35 cycles 68°C x 5 m 4 °C x Hold
AMA 1 2nd round 2hrs 30m	VM815/3: GGAACTCAATATAGACTTCC VM990: AATTCTTTCTAGGGCAAAC	95°C x 2.30 m 94°C x 30 s, 51°C x 30 s, 68°C x 45 s - 40 cycles 68°C x 5 m 4°C x Hold

Table 1: List of Primers sequences and cycling conditions.

Data interpretation

The MSP3_ K1 allele was identified as a single fragment of ~ 500bp in length and the MSP-3_3D7 allele as a single fragment of ~400 bp. Any other MSP-3 fragment different from the mainly allele K1 and 3D7 found was also reported.

For EBA-175, the CAMP allele (C fragment) was identified as a single fragment of ~714 bp while the FCR3 allele (F fragment) identified as a single fragment of ~795 bp relative to CAMP and FCR3 controls respectively. Mixed infections were defined for each marker as the simultaneous presence of K1 and 3D7 for MSP-3 or the F and C fragment for EBA-175 in the same sample.

The AMA-1_ K1, AMA-1_ 3D7 and AMA-1_HB3/7G8 were identified as a single fragment of 285 bp, 400 bp and 335 bp respectively when digested by MseI, SspI and BfuCI.

Statistical analysis

Allelic data were entered in Microsoft Excel format and exported to Epi Info 6.04a (http://www.cdc.gov/epiinfo/Epi6/EI6dnjp.html) for analysis. The data comparisons were made using the Chi square or Fisher's exact test, or Student's *t*-test data with a statistical significance threshold of $p<0.05$.

Ethical considerations

Ethical clearance from the Conseil National de Recherche en Santé (National Council for Health Research) in Senegal was obtained (SEN42/06), and a signed informed consent from the parent or the child legal guardian was required prior to blood sample collection.

Results

Patient characteristics

A total of 601 patients were screened. Out of these patients 170 were *P. falciparum* positive and meet the inclusion criteria. Patient characteristic are consign in Table 2.

	2006 (n=288)	2008 (n=313)	p-value
Malaria prevalence, n (%)	101 (35.0)	69 (22.0)	0.06
Sex, male n (%)	55 (54.4)	36 (52.1)	0.76
PD: Geometric mean	32383.9 ± 37362	31713.7 ± 37253	0.71
Range	1034-195764	1016-195837	

Table 2: Patient characteristics; PD: Parasite density.

PCR efficacy

Overall 170 *P. falciparum* mono-infection parasites isolated confirmed by microscopy and collected in Keur Soce area (Senegal) were used for DNA extraction. Out of these, 101 samples were collected in 2006 and 69 in 2008.

Out of the 170 *P. falciparum* isolates, 79% [135/170; n=77 (2006); n=58 (2008)], 82% [140/170; n= 83(2006); n=57 (2008)] and 75% [128/170; n=76 (2006); n=52 (2008)] were successfully analysed for Eba-175, MSP-3 and AMA-1, respectively. PCR negative samples were excluded from further analysis.

Size polymorphism and alleles frequencies

At each locus different types of alleles found were analysed to assess their frequencies. Based on the molecular weight of amplicons, the isolates were classified according to the two allelic type of EBA-175 (F-loop and C-loop), the two allelic type of MSP-3 (K1 and 3D7) and the three allelic types of AMA-1 (K1, 3D7 and HB3).

The analysis of the EBA-175 dimorphic alleles showed that F-Loop was predominant [EBA-175 F_loop (62.3%), EBA-175 C_loop (46.1%)] ($p=0.008$) Figure 1. In addition of the two main alleles, we noted frequencies of 15% and 5% for EBA-175 400 pb and EBA-175_360 pb respectively in the study area.

For MSP-3 alleles results showed frequencies of 49.2% and 54.2% for K1 and 3D7, respectively. In addition, excepted the high frequency observed in MSP3_350 bp allele (15%), no statistically significant difference was observed in the major (K1 and 3D7) MSP-3 alleles frequencies (p=0.40).

For AMA-1 pattern, results showed a comparable frequency between the three main AMA-1 alleles identified in this study. We found 39%, 33% and 32% for AMA-1_K1, AMA-1_Hb3 and AMA-1_3D7 alleles respectively. No significant difference was observed in the AMA-1 allele frequencies (p=0.46)

Plasmodium falciparum genetic diversity

The results showed that the EBA-175 gene presented 4 different alleles identified. The two main alleles [F loop (60%) and C_loop (46%)] are more frequent. But the other alleles found (EBA-175_400 bp, EBA-174_360 bp) had frequencies higher than 5% in the parasite population (Figure 1).

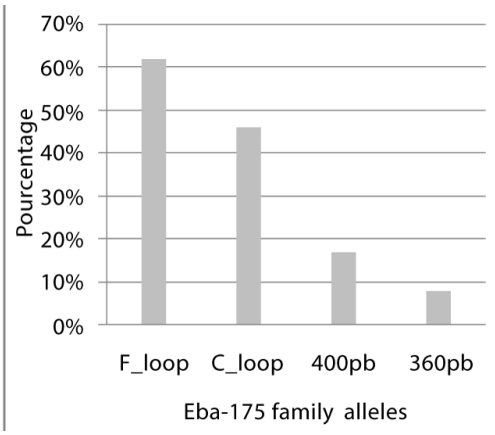

Figure 1: Allele frequencies of EBA-175.

Regarding the MSP-3 patterns, the analysis revealed the presence of 3 different alleles in Keur Soce study population: MSP-3_K1 (50%), MSP-3_3D7 (54%) and MSP-3_350 pb (14%) (Figure 2).

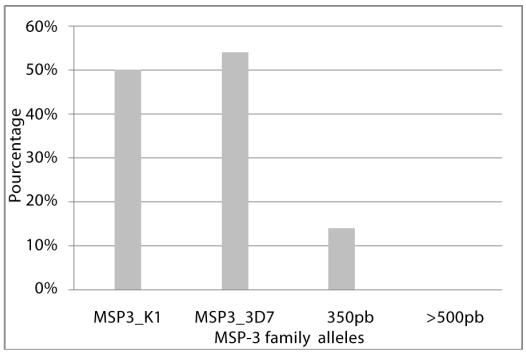

Figure 2: Allele frequencies of MSP-3.

For AMA-1 pattern, the three main alleles are found. Analysis of AMA-1 alleles showed comparable frequencies between the three main alleles found. More than 30% for all AMA-1 alleles identified in our study [AMA-1_K1 (39%), AMA-1_3D7 (32%) and AMA-1_HB3 (33%)] was found (Figure 3).

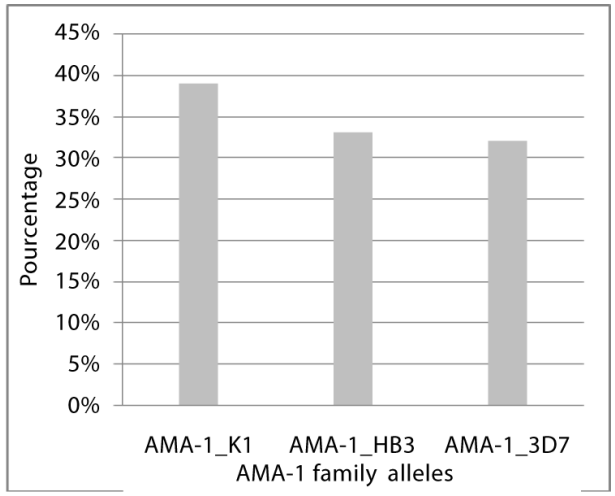

Figure 3: allele frequencies of AMA-1.

Haplotype frequencies over time

For EBA-175 pattern, the haplotype single allele was more frequent than the haplotype with two or three alleles over year (Table 3). The difference was significant from 2006 to 2008 (p=0.003) while EBA-175 haplotype two alleles, showed no significant difference between 2006 and 2008 (p=0.020). The haplotype with three alleles was missed in 2008.

	2006	2008	P-value
EBA-175_1allele	51 (66.23%)	47 (88.68%)	0.003
EBA-175_2alleles	22 (28.57%)	6 (11.32%)	0,020
EBA-175_3alleles	4 (5.20%)	0 (0.00%)	

Table 3: frequency of different haplotypes of EBA-175.

Regarding MSP3 pattern, the MSP-3_1 allele was also more frequent than the MSP-3_2 alleles with no significant difference between 2006 and 2008 (p=0.028) (Table 4). For haplotype MSP-3_2 alleles, results showed that, these haplotype was more frequent in 2006 than in 2008 with no significant difference (p=0.028). The MSP-3_3 alleles were missed in our study.

	2006	2008	p- value
MSP3_1allele	66 (79.5%)	53 (92.9%)	0.028
MSP3_2alleles	17 (20.5%)	4 (7.0%)	0.028

Table 4: frequency of MSP-3 haplotypes.

Discussion

The malaria parasite was shown to exhibit extensive genetic diversity, particularly in the surface antigens that have been under selective pressure and historically considered as the targets of subunit

vaccines. Unfortunately, this extreme genetic diversity poses a big challenge for an effective vaccine development as it could lead to "vaccine -resistant malaria" with non-vaccine type parasites in vaccinated individual [8]. Therefore, to make the vaccine more efficacious against any natural *P. falciparum* infection, multiple allelic forms of an antigen may need to be incorporated in a vaccine formulation [10,25].

The present study based on the assessment of the genetic diversity of malaria vaccine candidates (EBA-175, MSP-3 and AMA-1) in Keur Soce area (Senegal), provides a pre-vaccine, baseline data of the distribution of major allelic classes.

Here the study reported evidence of the presence of the main allele types of EBA-175 (F-loop and C-loop), MSP-3 (K1 and 3D7) and ama-1 (K1, 3D7 and HB3) at high frequencies. But when looking at the individual loci and the corresponding alleles, it emerges clearly that substantial differences exist in the allelic distributions between other countries. Overall, for eba-175 gene, our results showed that the F-loop was the predominant allele. These results were similar to the previous observations made in comparable area [26]. This trend in EBA-175 allelic form distribution in keur socé confirmed the previous report in Central and Southern Senegal [25].

The analysis of the polymorphic sequence of MSP-3 gene which present many amino acid substitutions leading to the two major allele types (K1 like and 3D7 like) [27] is of interest to determine how a vaccine candidate antigen that is polymorphic in nature and under immune selection was distributed in such malaria endemic area. These two major allelic types showed comparable frequency in our study area. In addition to the major dimorphic alleles (K1 like and 3D7 like), the presence of MSP-3_350 bp in our study showed that MSP-3 gene is a polymorphic gene [28]. The predominance of the two major allele types (K1 like and 3D7 like) noted in our study confirmed previous studies results showing that the major dimorphic alleles of MSP-3 were maintained in the species, intact within populations with low level of recombination [29].

Regarding AMA-1 gene, *P. falciparum* isolates from our study belonged to three (3D7, K1 and HB3) of the four groups of alleles previously identified based on the hypervariable region (HVR) of AMA-1(4). The fourth group alleles was not identified because in our study we used only three restriction enzymes, specific to the first three groups of alleles for which positive controls were available during the PCR-restriction fragment length polymorphisms analysis. Limited data exists for AMA-1 four group's allele's distribution in Sub-Saharan Africa. In the present study results showed comparable frequency between the three AMA-1 alleles. Our results showed a comparable frequency of the three AMA-1 alleles (AMA-1_HB3, AMA-1 _K1 and AMA-1_3D7) compared to Ghana and Burkina Faso [30]. The low frequency observed in AMA-1_3D7 in Ghana and Burkina [30], could probably be explained by geographical distibution, positive natural selection as it was previously suggested. In general the AMA-1 diversity was low and comparable in many countries even there were some regional differences reported on the prevalence of the different allelic groups which difference may probably pose a problem for a vaccine based on this antigen. Although a remarkable conservation of the AMA-l molecules were previously reported [11], it is very important to understand the extent to which variation in AMA-1 gene may compromise vaccine development.

Conclusion

The main allelic families found in other African countries were also found in Keur Soce area. These major allelic families circulating (in Keur Soce area should be considered for the successful malaria vaccine in Senegal.

Conflict of interest

No conflict of interest was noted.

Author's contribution

MN conceived the study, designed the experiments and carried out the molecular genetic analysis. BF supervised the study and corrected the manuscript. RT performed the statistical analysis. AL and AA participated on samples collection. OG coordinated the study and provided conceptual advice. All the authors read and approved the final manuscript.

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